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The reduction of *Legionella* spp. in water and in soil by a citrus plant extract vapour

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Running title: Effectiveness of a citrus EO vapour against *Legionella* spp. in soil and water.

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Abstract

Legionnaires disease is a severe form of pneumonia caused by *Legionella* spp. often isolated from environmental sources including soil and water. *Legionella* spp. are capable of replicating intracellularly within free living protozoa, once this has occurred *Legionella* spp. is particularly resistant to disinfectants. Citrus Essential Oils (EOs) vapours are effective antimicrobials against a range of microorganisms, with reductions of 5 log cells ml^{-1} on a variety of surfaces. The aim of this investigation was to assess the efficacy of a citrus EO vapour against *Legionella* spp. in water and in soil systems. Reductions of viable cells of *Legionella pneumophila*, *Legionella longbeachae*, *Legionella bozemanii* and *intra-amoebal culture of Legionella pneumophila* (water system only), were assessed in soil and in water after exposure to a citrus EO vapour at concentrations ranging from 3.75 mg/l air to 15g/l air. Antimicrobial efficacy via different delivery systems (passive and active sintering of the vapour) was conducted in water and GC-MS analysis of the antimicrobial components (linalool, citral and β-pinene) determined. There was up to a 5 log cells ml^{-1} reduction in *Legionella* spp. in soil after exposure to the citrus EOs vapour (15 mg/l air). The most susceptible strain in water was *L. pneumophila* with a 4 log cells ml^{-1} reduction after 24 hrs via sintering (15 g/l air). Sintering the vapour through water increased the presence of the antimicrobial components, with a 61% increase of linalool. Therefore, the appropriate method of delivery of an antimicrobial citrus EO vapour may go some way in controlling *Legionella* spp. from environmental sources.

Introduction

In 2011, 4 897 cases of Legionnaires disease were reported by EU member states and Norway and Iceland, with six countries (France, Italy, Spain, Germany, Netherlands and the United Kingdom) contributing to 83% of all the cases[1]. In the same year 239 cases were reported by the National Surveillance Scheme in England and Wales, with the number of cases steadily increasing since the mid-1990s when, on average, between 110 and 160 cases per annum were recorded [2].

Legionnaires disease is a severe form of pneumonia [2,3] caused by the Gram-negative, aerobic rod *Legionella* spp., It mainly affects the elderly and immune-compromised people and is more generally reported in men [3,4]. *Legionella pneumophila* is the predominant human pathogenic strain and is responsible for about 90% of all human infections by *Legionella* spp. [5,6]. Sixteen serotypes of *L. pneumophila* exist, but serotype 1 is the most important clinically [5]. An international collaborative survey showed that 84.2% of all isolates in patients with community acquired Legionnaires disease were serotype 1[7]. However, *Legionella longbeachae* and *Legionella bozemanii* were also isolated at rates of 3.9 % and 2.4 % respectively [7]. However incidence rates vary from country to country and also from source to source. For example, *L. longbeachae* is the most commonly isolated species from patients in Australia [8], accounting for about 30% of *Legionella* isolates from Australia and New Zealand. A recent study by Currie et al (2013)[9] in the UK has shown that 15 out of 24 compost samples positive for *Legionella* spp. with *L. longbeachae* being the most commonly isolated. In one study, *L. longbeachae* was found in the sputum of a patient who had been in contact with potting soil [6], and it was suggested that aerosol-aided spread and evaporation of water in the potting soil were the possible routes of transmission [6,10]. Legionella have also been isolated from waste management facilities dealing with unwashed solid articles, probably via exposure to soil [11].

The natural habitat of *Legionella* is fresh water such as lakes and rivers [3] where it grows planktonically or in biofilms, with an optimum temperature range for growth and survival of between 30°and 40°C. However, it can enter man-made water systems and survive thus creating a potential source for infection. Previous studies have isolated the bacterium from drinking water systems, cooling towers of air conditioning units, whirlpools, spas, fountains, ice machines, vegetable misters, dental devices and shower heads [12]. Infection in humans occurs via inhalation of an aerosolised form of *Legionella* spp. from a contaminated source or via aspiration of contaminated water, which can occur within milliseconds [5,6,10]. There are no specific standards in the UK for acceptable levels of *Legionella* spp. in water, however, there is a statutory requirement that the owners of buildings that have equipment predisposed to *Legionella* spp. must ensure that the equipment is maintained to prevent the growth and spread of the organism [13].

Legionella spp. are also capable of invading and replicating intra-cellularly within free living protozoa [3,12]. *Acanthamoeba polyphaga* is the most common host of *Legionella* spp. in natural environments [3]. Free-living amoebae are capable of forming cysts which confers resistance to extreme temperatures, desiccation and disinfection [14] and also provide protection to the intracellular *Legionella* cells hence making them more able to survive similarly unfavourable conditions. Furthermore, several studies have shown that *L. pneumophila* exhibits a higher stress resistance and is more invasive and virulent, after it has replicated within a protozoa cell [3,12,15]. It has been suggested that *Legionella* cells invade and grow within human macrophages in a similar way as they do within protozoan cells[16].

Commonly chemical disinfectants or biocides are used to prevent microbial contamination and growth of prospective pathogenic microorganisms in man-made aquatic sites[14]. However, they are only effective in high concentrations which tend to be harmful to humans and thus the use of natural alternatives to these chemicals may reduce risk of toxicity. Citrus

Essential Oils (EOs) were first noted for their antimicrobial effect in 1949 by Piacentini [17]. In contrast to chemical disinfectants citrus EOs are "Generally Recognised As Safe" (GRAS) and therefore are acceptable for use in food and water systems.

In recent studies, the treatment of a range of pathogenic bacteria including MRSA, both vancomycin-susceptible and vancomycin-resistant strains of *Enterococcus faecium* and *Enterococcus faecalis* have been shown to be susceptible to the vaporised form of the unique blend of the citrus essential oils at a concentration of 15mg/l air [18, 19]. Furthermore, the citrus EO vapour used in this study has been shown to be effective against the foodborne pathogens *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli* O157 and *Campylobacter jejuni* [20, 21]. However, to date, the studies on the antimicrobial nature of the citrus EO vapour have only tested its effectiveness on surfaces such as stainless steel and other food surfaces and not against microorganisms in liquid systems because of the hydrophobic nature of its components. In addition, the use of EOs in water is not very effective because their vapours mainly consist of phenolic compounds which have poor solubility resulting in a reduced antimicrobial activity. Water also reduces volatility as compounds with hydroxyl groups may be more solvated and remain in water phase [22]. Previous studies using a bio-autography method followed by Atmospheric Pressure Chemical Ionisation (APCI-MS) and SPME GC-MS have shown that there is a favourable release of the active compounds (linalool, citral and β-pinene) from the citrus EO vapour which facilitates the antimicrobial activity [23].

The aim of this study was to investigate the effectiveness of the antimicrobial citrus EO vapour against *Legionella* spp. in soil and to establish if sintering is effective as an active delivery system against *Legionella* spp. and intra-amoebal *L. pneumophila* in water.

Methods

All investigations were carried out in duplicate on at least three separate occasions.

Micro-organisms and Culturing Methods

Legionella pneumophila (ATCC 33152), *Legionella longbeachae* (ATCC-33462) and *Legionella bozemanii* (ATCC 33217) were grown on *Legionella* CYE agar base (CM0655) supplemented with *Legionella* BYCE growth supplement (SR0110C) at 37°C for 48 hrs. *Acanthamoeba polyphaga* (CCAP 1501/14) was cultured using Peptone Yeast Glucose (PYG) medium (10g proteose-peptone, 0.5g yeast extract, 0.1M glucose, 25ml Page's Amoebal saline Solution (PAS) 1 , 25ml PAS 2, 450ml water) adjusted to pH 6.5 with KOH. PAS solutions [24]. Aliquots of 1 ml of *A. polyphaga* cultures were suspended in 5ml PYG medium in tissue culture flasks. The protozoa cultures were incubated for three days at 35°C.

Preparation of Acanthamoeba polyphaga for co-culture experiments

PYG broth (22 ml) was inoculated with 2 ml of a three day *A. polyphaga* culture. The culture flasks were incubated horizontally for three days at room temperature.

After incubation flasks were shaken to remove the protozoa from their surface. The sample was centrifuged at 400g (HettichRotanta 460 S Tuttlingen, Germany) for 6 min at room temperature. The pellet was then washed twice in 20 ml of PAS and re-suspended in 15 ml of amoebal saline. Cell counts were obtained using a haemocytometer (Thoma, Hawsley London, 0.1 mm, 1/400 mm^2). Co-culturing required a final concentration of 10^5cells ml^{-1} .

Intra-amoebal culture of Legionella pneumophila

A suspension of 10 ml *A. polyphaga* (10^5 cells ml^{-1}) was mixed with a suspension of 10 ml *L . pneumophila* (10^2 cells ml^{-1}) in a tissue culture flask and incubated at 35°C for 10 days. The sample was then centrifuged at 400g for 6 min at room temperature to remove the protozoa. The supernatant was subsequently centrifuged at 2080g for 15 min at room temperature and

discarded. The pellet was washed twice with 20 ml PAS. The resulting suspension (10^5cells ml^{-1}) was then mixed with 20 ml of a fresh three day-old culture of *A. polyphaga* (10^5cells ml^{-1}) and incubated again at 35°C for three days. The wash steps were then repeated. The final pellet was re-suspended in 20 ml PAS, with a final concentration of *Legionella* of 10^8 cells ml^{-1}.

Citrus EO vapour and vapour components

The citrus EO blend consisted of orange (*Citrus sinensis*) and bergamot (*Citrus bergmia*) essential oils (Belmay, Northampton, UK) in a 1:1 (v/v). Limonene 97%, (18, 316-4), linalool 97% (W26, 350-8), citral 95% (C8, 300-7), β-pinene 99% (402753) were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK).

The assessment of a citrus EO vapour and its components against Legionella spp. in soil

Potting soil (potting mix, Miracle Gro, Scotts, UK) was sterilised and 1.5 g placed in a petri dish in a 1000 ml beaker. The soil was inoculated with either 400 µl of *L. pneumophila, L. longbeachae* or *L. bozemanii*. Filter papers (Whatman disks, 2cm) were impregnated with the EO mix to give final concentrations of either 3.75 mg/l air, 7.5 mg/l air or 15 mg/l air and sealed with parafilm (FIL1026, Scientific Laboratory Supplies, UK). The beakers were incubated for 24 h at 37 °C. The soil was then placed in 30 ml of maximum recover diluent (MRD), vortexed for 2 min., spread plated onto CYE agar, incubated for 48 hrs at 37°C and counts obtained. Controls were inoculated soil samples not exposed to the citrus EO vapour or components.

Survival of Legionella spp. in water after exposure to the citrus EO vapour

Passive Exposure

Cells of either *L. pneumophila*, *L. longbeachae* or *L. bozemanii* or *L. pneumophila* that had been passaged through *A. polyphaga* were inoculated into sterile water in 1L beakers to give a final concentration of 10^7 cells ml^{-1} . Filter papers impregnated with the citrus oil to give final concentrations of 3.75 mg/l air, 7.5mg/l air, 15mg/l air, 150mg/l air or 15g/l air in the atmosphere were placed in the beaker, which was sealed and incubated at room temperature for 24 h. After exposure 100 µl samples were spread plated on CYE agar and incubated at 37°C for 48 hrs and colonies counted. Controls were water samples not exposed to the citrus EO vapour.

Active Exposure

A cylinder filled with compressed air was connected to a 500ml vacuum flask (headspace: 590 ml), containing either 150 mg/l air , 820.5 mg/l air or 1500 mg/l air of citrus EO vapour. A sinter (10 micron pores, Sigma Aldrich, UK), running from the vacuum flask was then placed into a 100ml conical flask containing 100ml water and *L. pneumophila* at a final concentration of 10^6 cells ml^{-1} (Figure 1), the sinter forces the air containing the EO through micron size pores into the water creating small bubbles which continuously move through the water sample. The citrus EO vapour was left to equilibrate in the vacuum flask for 15 min before the air flow (0.225 L/min) and the heating plate (30°C) were switched on. Samples were removed at 0, 1, 2, 4, 6 and 24 h after starting the air flow, spread plated onto a CYE agar plate in triplicate, incubated at 37°C for 48 hrs and colonies counted.

The investigations were repeated using either *L. longbeachae*, *L. bozemanii* or an intra-amoebal culture of *L. pneumophila* and the concentration of citrus EO vapour shown to be the most effective against *L. pneumophila*. Controls were cells exposed to pure air flow.

GC-MS Analysis

To quantify the active antimicrobial components (linalool, citral and β-pinene) in the passive and active exposure, the same apparatus as described above was used, but without microorganisms added to the water. Additionally, the initial amount of citrus EO was increased by 10 fold (15g/L) to enable detection. GC-MS analysis was undertaken on the citrus EO vapour in water or in ethanol without any further sample preparation. Experiments were performed for 24hrs in the case of water, and 4 hrs for ethanol due to volatility limitations (Table 1). All experiments were performed in duplicate.

The GC-MS analyses were performed using a Bruker 450GC and 300-MS SQ mass spectrometer operated in EI mode at 70eV. A sample volume of 1μL with split ratio of 10:1 was injected at an inlet temperature of 250°C. The carrier gas was helium and maintained at a constant flow rate of 1.0 mLmin^{-1}. The gas chromatograph was equipped with a FactorFour VF-5MS capillary column (30m long × 0.25mm ID) with 0.25 μm film thickness. The temperature of the column was held at 40°C for 2min, ramped to 70°C 10°C min^{-1}, hold for 5 min, ramped to 150°C at 5°C min^{-1}, hold for 1 min and then ramped to 200°C at 10°C min^{-1}.

The MS ion source temperature was 180°C. Quantitative analysis was carried out using selected ion monitoring (SIM) mode at 70 eV. For each compound, the most abundant ions were selected from its spectrum. The chosen ions for SIM were 69, 84 and 152 for citral, 93, 69, 79 and 136 for β-pinene, 71, 93 and 154 for linalool. The limits of quantification for all three antimicrobial agents were 1.0 mg/l.

Results

210

211 The citrus EO vapour at concentrations as low as 3.75mg/l air reduced *Legionella* spp. by
212 between 0.5-1.5 log cells ml^{-1} in soil, however, when this concentration was increased to that
213 of 15mg/l air which has previously been shown to be effective against a range of different
214 microorganisms on surfaces, up to a 8 log cells ml^{-1} reduction was observed against *L.*
215 *bozemanii* compared to 1.53 log cells ml^{-1} and 0.7 log cells ml^{-1} $log_{(10)}$ for *L. longbecheae* and
216 *L. pneumophila* respectively (Table 2).

217 When water inoculated with *Legionella* cells was passively subjected to the citrus EO vapour
218 no reductions in counts were observed at 3.75 mg/l air, 7.5 mg/l air, 15mg/l air or 150 mg/l
219 air. However, when subjected to 15 g/l air a 2 log cells ml^{-1} reduction occurred for *L.*
220 *longbeachae*, although the other strains were unaffected (results not shown).

221 Actively sintering the citrus EO vapour into water inoculated with *L. pneumophila* resulted in
222 reductions over 24hrs of 1.5 log cells ml^{-1} and 4.5 log cells ml^{-1} ($p \leq 0.05$) for 150mg/l air and
223 1500 mg/l air respectively (Figure 2). These concentrations are 10-100 fold higher than that
224 previously shown (15mg/l air) to reduce microorganisms on surfaces such as stainless steel
225 (Fisher and Phillips, 2009a)

226

227 A reduction in cells numbers of *Legionella* spp. in water treated with a citrus EO vapour
228 (15g/l) through a sintering system is observed at 2 hrs exposure with reductions of between 1
229 - 2 log cells ml^{-1}. *L. pneumophila* was the most susceptible with a 4 log cells ml^{-1} ($p \leq 0.05$)
230 reduction in cell numbers at 24 hrs while *L. bozemanii* and *L. longbeachae* were reduced by
231 2.8 log cells ml^{-1} and 2.2 log cells ml^{-1} respectively. However, the vapour only had a
232 minimal effect on the co-cultured *L. pneumophila* with a 1.24 log cells ml^{-1} reduction in cell

numbers over 24 hrs (Figure 3). There was no significant difference ($p \leq 0.05$) between the active and passive systems of delivery of the citrus EO vapour against *L. longbeachae* where a 2 log cells ml^{-1} reduction was observed in both systems.

Only linalool was detected in water when 15g/l citrus EO vapour was passed through either passively or through the sintering system (active diffusion) which can be attributed to the relatively high water solubility of linalool (see Table 1). As shown in Figure 4, there was no significant difference in linalool content of water between the passive and active systems with concentrations of 24.9 mg/l and 26.5 mg/l linalool respectively after 45 min exposure. From 1 hr onwards a significant difference ($p \leq 0.05$) in concentration of linalool in the water was noted. After 24 hr exposure, the linolool concentration was 35.43 mg/l and 57.17 mg/l in the passive and active systems respectively, making the linalool content in the active system 61% more than that in the passive system.

When ethanol was the solute, no linalool was detected within the first 30 mins in solution either when the citrus EO vapour was diffused passively or by active sintering. After 4 hr the linalool content was 46% higher in the active system (Figure 5). This trend of higher linalool concentration in solution in the active system is similar to that observed when water was used as the solute and this is also the case for both citral and β-pinene. However, citral showed the highest difference with up to 2.35 fold more in the active systemafter 4 hrse.

Discussion

Overall the citrus antimicrobial vapour was active against *Legionella* spp. However, the extent of its efficacy was dependent on strain and substrate. In soil the vapour was most effective against *L. bozemanii* at 15mg/l air (Table 2) with a 7.88 log cells ml^{-1} reduction, there was no significant difference in reductions between the controls and 15mg/l air of citrus EO vapour against *L. longbeacheae* and *L. pneumophila,* demonstrating the vapour to have strain specific activity. *L. longbeacheae* is the most isolated *Legionella* spp. from potting soil in Australia (58%), with the rates of isolation of *L. pneumophila* being 13.3% [10]. Against both *L. longbecheae* and *L. bozemanii* the most effective concentration of the citrus EO vapour was 15mg/l air Similar results have been previously reported for *Enterococcus* spp. survival on a range of surfaces included, lettuce, cucumber and stainless steel with reductions of up to 5 log cells ml^{-1} [19,25].

The use of essential oils (EOs) as antimicrobials in water -based environments has not been explored in depth which is probably due to the lipophilic nature for the EOs and their relative insolubility in water. Traditionally when assessing EOs minimum inhibitory concentration, an agar dilution method is usually chosen above that of a broth dilution method for this very reason [6]. However, improvements to methodologies for the determining the antimicrobial efficacy of EOs in broth cultures with the use of emilsifiers have been made, making the assessment of EOs in aqueous solutions more effective[26]. The use of a sintering system to force the vapours of EOs through water eliminates the need for other emulsifying agents such as ethanol and Tween 80, thus increasing its potential use within equipment predisposed to *Legionella* spp. such as air conditioning units

The use of the vapours of EOs rather than EOs *per se* allows for single components to be targeted and analysed for their solubility and antimicrobial efficacy in water. Previous studies have shown that linalool, citral and β-pinene are the main antimicrobial components of the citrus EO vapour as determined by a bioautography method [23].

Figures 1-4 demonstrate that the use of a sintering system, thus forcing the vapours components through the water gives a greater reduction in *Legionella* spp. compared to natural diffusion of the components. The consequent reduction in cell numbers increased from zero in the passive system to 4.5 $\log_{(10}$ (Figure 2) in the active system against *L. pneumophila* at a citrus EO vapour concentration of 15g/l air. However, the concentration of the vapour needed to reduce the *Legionella* counts in water had to increase from 15mg/l air observed to be active in a soil system and other surfaces by 100 fold to 15g/l air when being sintered into water. There is limited published research on the effect of EOs in water environments. The use of buffered yeast extract broth with tween as an emulsifier, has been shown to be a suitable medium to assess Tea Tree EO activity against *Legionella* spp. [(26)]. and Chang et al. (2008)[(28)] assessed the use of cinnamon oil in hot spring water at a range of pHs with ethanol being used as an emulsifying agent. Minimum Bacterial Concentrations (MBCs) against *L. pneumophila* ranged from 400-1200 mg μl^{-1} with a contact time of 10 mins and 400-750 $\mu g\ ml^{-1}$ with contact time of 60 minutes.

The way in which the components are being passed through the water may also be crucial to the antimicrobial efficacy of the citrus EO vapour. Linalool, which has a higher solubility in water (1589mg/l at 25ºC) when sintered, is trapped within the water thus continuing to have an antimicrobial effect on the *Legionella* cells after 2 hrs exposure (Figure 4), resulting in an accumulation effect and in part may explain the 61% difference in concentration of the linalool between the passive and active systems at 24 hrs. However, both citral and β-pinene have a lower solubility in water (590 mg/l at 25ºC and insoluble respectively) and are not retained within the water when sintered as Figure 5, shows the amount of citral and β-pinene in the water at any given time as they pass through the water before they evaporate. This suggests that the antimicrobial effect of citral and β-pinene may be based on a collision process between the compounds and the *Legionella* cells as they pass through the water. The

increase in concentration of the antimicrobial compounds is noted from 2 hrs onwards (Figures 3 & 4), corresponding to a reduction in the *Legionella* cells in water from the same time point (Figure 3). The increase of the antimicrobial compounds after 2hrs is also noted in the vapour release intensity of headspace with a 0.5 log cells ml^{-1} increase linalool and β-pinene and 2 log cells ml^{-1} of citral [23].

Since there are no acceptable levels of *Legionella* spp. specified by the HSE in the UK, the maintenance of equipment that are pre-disposed to *Legionell*a spp. is the responsibility of the manager of the site/equipment, therefore, a range of different disinfectants and physical treatments are used including chlorine, monochloramine, UV and heat, all of which have drawbacks including rinsing, expensive equipment and running costs and are often not effective against inter-cellular *L. pneumophila.* The use of citrus antimicrobial vapour which is sintered through an enclosed water system such as air conditioning units and cooling towers may be a natural alternative that the FDA have deemed GRAS under general provisions of essential oils, oleoresins (solvent-free), and natural extractives [29]. The components identified to be antimicrobial (linalool, citral and β-pinene), are widely found in plants including fruits and herbs and often used within the food and fragrance industries. With Linalool having no recommended threshold limit value (TLV) or biological exposure index (BEI) and citral and β-pinene being listed by the International Fragrance Association (IFRA) as commonly being found in fragrances safe for use[30-32].

In conclusion this citrus EO vapour may be a potential solution to controlling *Legionella* spp. from environmental sources such as soil and water. The novel delivery system using sinters to force hydrophobic compounds through water could allow for the use of EO oil based products in new arenas.

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Table 1. Chemical properties of antimicrobial components in the orange: bergamot EO[22].

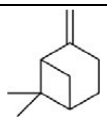
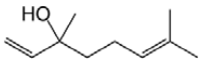
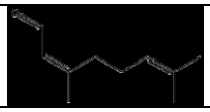
Name	Structure	Molar mass	Solubility in water	Partition coefficient (LogPow)
β-pinene		136.23 g/mol	Insoluble	5.4 at 25 ºC
Linalool		154.25 g/mol	1589mg/l at 25 ºC	2.97 at 23.5 ºC
Citral		152.23 g/mol	590 mg/l at 25 ºC	3.0 at 25 ºC

Table 2: Reduction of *Legionella* spp. in soil (mean ± SE: n=3) when exposed to a citrus EO vapour for 24hrs.

	L. longbecheae	*L. bozemanii*	*L. pneumophila*
Control	1.2 ± 0.2	1.05 ± 0.28	0.56 ± 0.22
3.75 mg/l	1.47 ± 0.18	1.42 ± 0.32	0.55 ± 0.23
7.5 mg/l	1.65 ± 0.16	1.71 ± 0.25	0.64 ± 0.24
15 mg/l	1.53 ± 0.22	7.88 ± 0	0.7 ± 0.41

Figure 1: Schematic diagram of the setup of active exposure of *Legionella* spp. to the vapour of a citrus EO vapour

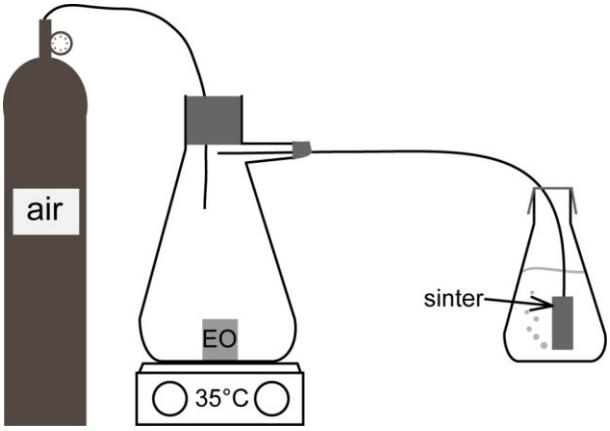


Figure 2: The mean survival of *L. pneumophila* when exposed to an antimicrobial citrus EO vapour in water via a sintering system. Control (exposed to air only) , 50 mg/l air, and 15 g/l air

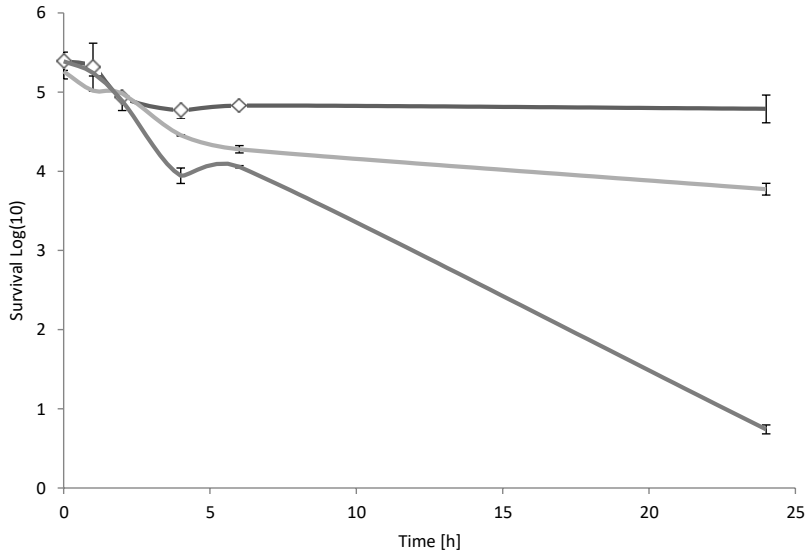


Figure 3: The mean survival of *Legionella* sp. when exposed to an antimicrobial citrus EO vapour (15g/l air) in water via a sintering system. *L. pneumophila* – –, *L. longbeachae* —, *L. bozemanii* — — co-cultured *L. pneumophila* — · —

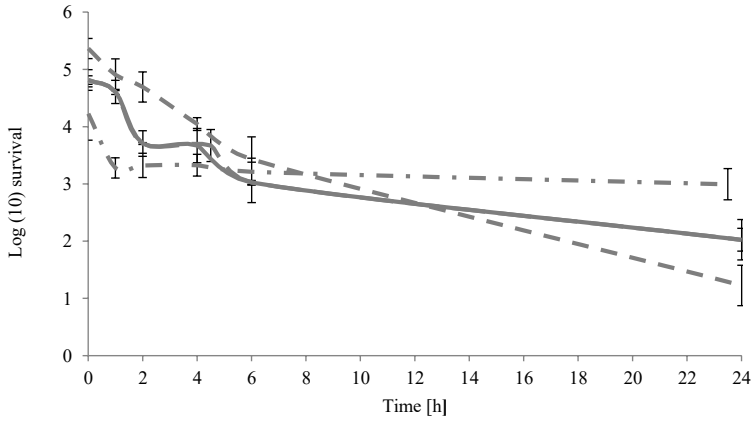


Figure 4. Linalool content in 100 ml water exposed to 15g/l antimicrobial citrus oil vapour in passive and active modes. Linalool-passive ◇ and Linalool-active ◆

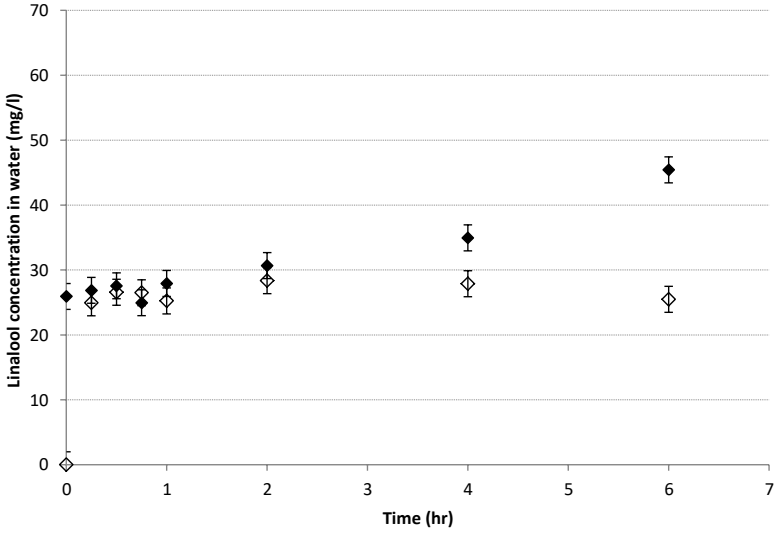


Figure 5. Antimicrobial agents in 100 ml ethanol exposed to 15g/l antimicrobial citrus oil vapour in passive and active modes. β-pinene-passive △ , β-pinene-active ▲, Linalool-passive ◇, Linalool-active ◆, Citral-passive ○ and Citral-active ●